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The study of process of alternative fuel production from renewable raw materials

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Abstract

The objective of the research was to study the process of biofuels production from raw materials of plant origin such as wastes of food and agricultural industry. The process includes a stage of enzymatic degradation of plant polysaccharides. The proposed method allows to produce ecologically friendly renewable biofuels and to provide partial utilization of carbohydrate-containing industrial wastes or by-products. The economic efficacy of alcohol production is increased due to the yield growth as a result of the optimization of the microbiological synthesis process.

The disadvantages of existing methods of biofuel production are usage of food crops as raw materials, high labour and financial costs incurred during planting, harvesting, handling and storage of such crops and a small yield of the final product. The proposed method of biofuel production is based on the usage of non-edible raw materials of plant origin such as wastes and by-products of isolation of protein preparations from lupin seeds. The bioconversion of polysaccharide complex of feedstock to soluble carbohydrates is carried out using a composition of hydrolytic enzymes with cellulase activity of at least 3500 units gram 1 and xylanase activity of at least 2500 units gram-1. The liquid biobutanol is produced by fermentation of carbohydrate substrate obtained as a waste or a by-product after processing of raw materials with bacteria *Clostridium beijerinckii*.

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Keywords: renewable energy; plant polysaccharides; multienzyme composition; solid biofuels; liquid biobutanol; *Clostridium beijerinckii*

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1. Introduction

Alternative energetics is a promising field for applications of biotechnology. The development of industrial methods of production of biofuels has been gaining attention in recent years. In particular, one of the perspective methods of producing acetone butanol ethanol (ABE) is a cultivation of *Clostridium beijerinckii* using different growth mediums [1, 2]. A number of organisms is available for this bioconversion such as *Clostridium beijerinckii* P260, *Clostridium beijerinckii* BA101, *Clostridium acetobutylicum* and *Clostridium saccharobutylicum* P262. These cultures can utilize both hexose and pentose sugars derived from lignocellulosic hydrolysates such as corn fiber, wheat straw, barley straw, maize stover and switchgrass. Substrates such as Jerusalem artichoke, maize, rye, millet, molasses, potato can be used by the cultures listed too [3]. At the same time with the prospects of the use of raw materials of plant origin for biofuel production it is also important to increase their yield [4].

The processing of raw materials of plant origin requires the use of environmentally friendly methods related to cost-effective usage of resources, namely, utilization of such wastes of industrial food processing as molasses, milk whey and others. The alternative raw materials may be mash, potato starch and products produced from non-traditional crops [5]. In particular, in Russia at the University of Chemical Technology named after D. Mendeleyev, the quality of by-products of production of concentrated protein products was evaluated, as well as an experimental validation of the possibility of usage of wastes of soy protein production in feed supplements was made [6]. Other promising resources of plant origin that can be used to produce biofuels are birch wood and algae [7, 8].

Different species of lupin seeds have been gaining popularity in food technology as a raw material for extraction of protein and lipid fractions. Lupin protein preparations have been used in meat and bakery industries [9–11], and in the production of dairy analogues [12]. Lupin seeds of certain species have been used for solvent extraction of oil by means of different solvents [11]. Lupin protein isolates preparations were obtained from narrow leaf lupin seeds [13, 14].

This work is focused on the development of a perspective method of using food industry wastes with a high content of structural polysaccharides for motor fuel production. The method deals with utilization of a by-product of lupin protein isolate (LPI) production such as deproteinized lupin meal residue (DLMR). The bioaccessibility of DLMR can be increased through a chain of sequential processes, involving acid treatment and biodegradation with a specially selected multi-enzyme composition.

Enzyme compositions with different substrate specificity are often applied in industrial biotechnology. For instance, to increase the bio-accessibility of feeds, different compositions based on cellulase, xylanase, mannase and other enzymes have been developed [15, 16]. In the beer manufacturing process, a variety of compositions of amylase and cellulose are widely used at the mash preparation stage [17].

The objective of this study was to develop a waste-free technology of production of lupin protein isolate, involving mechanical and enzymatic degradation of solid waste materials with formation of the growth medium and its subsequent fermentation by *Clostridium beijerinckii*.

Nomenclature

ABE	acetone butanol ethanol
DLMR	deproteinized lupin meal residue
LPI	lupin protein isolate
UHT	ultra heat treatment
HPLC	high-performance liquid chromatography
m.f.b.	moisture free basis

2. Material and methods

2.1. Materials

Lupin flour from dehulled seeds of *Lupinus angustifolius* (crude protein – 51 % on moisture free basis (m.f.b.), crude fat – 7 % on m.f.b., crude fiber – 2.9 % on m.f.b.) was obtained from All-Russian Scientific Research Institute of Lupin, Bryansk. *Clostridium beijerinckii* was used for bioethanol production tests (obtained from All-Russian Institute of Agricultural Biotechnology). Five enzyme preparations were used, see Table 1.

Table 1. Enzymes preparations.

Enzyme preparation	Characteristics	Enzyme activity	Manufacturer
Celluclast BG	carbolytic enzyme preparation produced by submerged fermentation of the selected strain of fungus *Trichoderma reesei*	3500 endoglucanase units gram^{-1}	Novozymes, Denmark
Pentopan Mono BG	xylanase preparation from fungi *Aspergillus oryzae*	2500 fungal xylanase units gram^{-1}	Novozymes, Denmark
Amylosubtilin	amylase preparation from *Bacillus subtilis*	950 fungal amylase units gram^{-1}	Sibbiofarm, Russia.
Cellolux-F	cytolyticcomplex enzyme preparation produced by submerged cultivation of fungus *Trichoderma viride*	2000 cellulase units gram^{-1}, 8000 fungal xylanase units gram^{-1}, 1500 endoglucanase units gram^{-1}	Sibbiofarm, Russia
Cellolux-A	cytolyticcomplex enzyme preparation of fungi origin	2000 cellulase units gram^{-1}, 8000 fungal xylanase units gram^{-1}, 1000 endoglucanase units gram^{-1}	Sibbiofarm, Russia

2.2. Production of lupin protein isolate

The process of production of lupin protein isolate was based on a separation of protein substances from other seed components similar to a traditional industrial technology of production of soy protein isolates from soybean meal [18]. Grinded lupin seeds, lupin meal and lupin flour could be used as a raw material for isolation of lupin proteins.

At the first stage, the alkaline extraction is used for isolation of soluble proteins and nitrogen-free extractable compounds such as soluble carbohydrates, organic acids, vitamins, etc. Then the liquid phase is separated from the insoluble residue, hereinafter (DLMR). The residue could be washed additionally by water (2nd stage extraction) to remove residual quantities of soluble compounds, see Fig. 1.

At the second stage, proteins are recovered from the liquid phase by precipitation. For this purpose acidic precipitation at the protein isoelectric point is used and then, the series of separations of solid and liquid phases and washing stages are performed. After protein precipitation lupin whey is separated, it contains protein-free soluble compounds mainly soluble carbohydrates. Then there are stages of neutralization, pasteurization and spray drying of protein isolate.

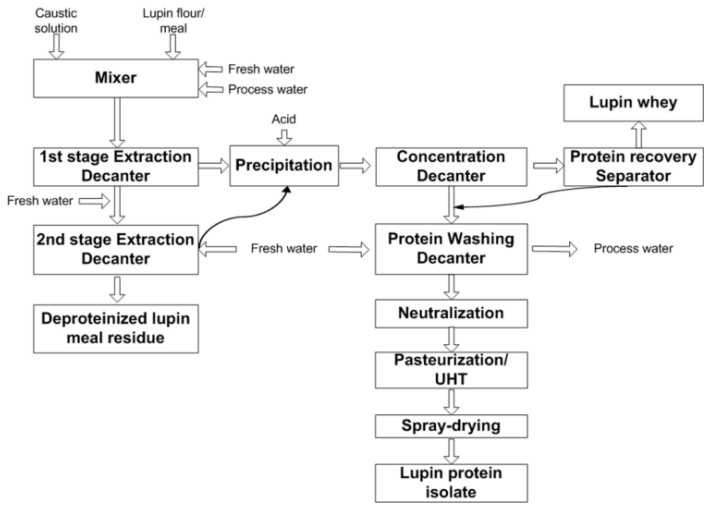

Fig. 1. Flow diagram of production of lupin protein isolate.

2.3. Obtaining ofDLMR

The alkaline extraction of lupin flour was carried out at hydromodule of 1:10; pH was adjusted to pH 9.5 by adding of 0.1 % NaOH solution. The process was carried out in a thermostatic vessel with a magnetic stirrer for 30 min at temperature of 50 ºC. Then the liquid phase was separated and the solid residue was washed with fresh water at ratio 1:5 for 30 min. The mechanical separation of phases was performed both times by centrifugation at 4000 × g for 30 min. The final solid residue obtained after the 2nd centrifugation was named asDLMR. Total content of carbohydrates, crude protein content, crude fiber and crude fat of DLMR were analyzed.

2.4. Pretreatment of DLMR and fermentation with C. Beijerinckii

DLMR was subjected to consecutive acid and enzymatic hydrolysis prior fermentation with *C. Beijerinckii.*

DLMR was mixed with water at ratio 1:10 at constant agitation, acidified with 5 % hydrochloric acid to pH 1.5 and then subjected to heat treatment at temperature 120 °C in a high-pressure steam sterilizer for 60 min. Principle process diagram is presented in Fig. 2. The resulted suspension was cooled to room temperature and transferred into a sterile fermenter BioFlo 115 (New Brunswick, Eppendorf).

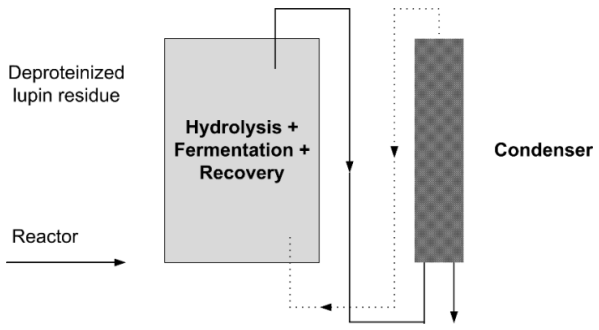

Fig. 2. Principle process diagram of production of biobutanol or ABE from deproteinized lupin meal residue in the integrated system.

The pH level of the mixture was adjusted to 6.5 with 10 M NaOH solution; 10 ml of yeast extract per 1 l and vitamins, minerals, and buffer were added. To create anaerobic conditions oxygen-free N_2 gas was added and agitated

at 150 rpm [1]. To increase the bioavailability of substrate substances, the suspension was subjected to enzymatic treatment using enzyme complex comprised of enzyme preparations Celluclast (1.1 ± 0.2 units $gram^{-1}$), Pentopan Mono (5.2 ± 0.4 units $gram^{-1}$), Amilosublilin (2.5 ± 0.2 units $gram^{-1}$) at temperature 35 °C for 40 min [13]. In the course of the experiments, doses of multi-enzyme composition and complex cytolytic systems ranged from 1 % to 2 % of substrate weight. After enzymatic treatment agitation and N_2sparging were stopped. At the next stage, activated culture of *C. Beijerinckii* (70mL) was added. Fermentation was carried out at 35 ºC which was achieved by means of water circulation. ABE vapors were condensed and collected in a receiver.

2.6. Measurements

Moisture content of DLMR was determined by the gravimetric method [19]. Crude protein content was analyzed by Kjeldahl method at the automated analyzer Kjeltec Auto 1030, Sweden, according to the standard protocol of the manufacturer. The conversion factor used to estimate protein content was Nx 6.25. The crude fat was determined by Soxhlet method at automated analyzer SER 148 (VELP Scientifica, Italy) according to the standard protocol of the manufacturer. The ceramic fiber filter method was used to determine the crude fiber [20]. Analysis of the total content of water-soluble carbohydrates was conducted by Bertrand method [21]. Changes in pH were measured with Orion 920A pH-meter (Russia). For the preparation of the nutrient medium, the steam sterilizer UT-4735 (ULAB) was used. Component analysis of mono- and disaccharides was conducted on HPLC analyzer 'Stayer' (Akvilon, Russia) with refractometric detector. The separation was obtained using a mobile phase consisting of acetonitrile and water in ratio of 77:23 using the column 'Luna NH_2 5μ', (Phenomenex, USA). ABE productivity was calculated as total ABE (present in the reactor plus condensed) in g L^1 [1].

2.6. Statistical evaluation of the data

All experiments were performed with at least three replicates; data was processed by methods of mathematical statistics at theoretical frequency 0.95. Statistical processing of data was carried out using computer programs Microsoft Office Excel 2010 and Mathcad 15.0.

3. Results and discussion

3.1. Characteristics of DLMR

The study showed that the content of main nutrients in the meal residue was influenced by the efficacy of the alkali extraction of proteins and soluble compounds and by washing stages (Table 2). The residue is a paste-like product with a high moisture level (higher than 80 %) unstable during storage.

For comparison, a deproteinized soy meal residue produced after isolation of soy proteins from soybean meal (white flakes) was also analyzed. However, the composition of the soy by-product is different to some extent from the lupin residue.

Table 2. Chemical composition of DLMR.

Type of waste	Moisture, %	Crude ash, % m.f.b.	Crude fiber, % m.f.b.	Crude protein, % m.f.b.	Crude fat, % m.f.b.	Other carbohydrates (by difference), %
DLMR(after alkali extraction)	83.1 ± 1.2	6.1 ± 1.2	14.3 ± 1.0	24.1± 1.2	3.9 ± 0.5	51.6
DLMR (after water washing)	81.1 ± 1.4	5.2 ± 1.2	15.2 ± 1.1	22.4 ± 1.1	3.3 ± 0.5	53.9
deproteinized soybeen meal residue (after alkali extraction)	82.5 ± 1.2	4.1 ± 1.1	15.3 ± 1.2	20.1 ± 1.3	1.3 ± 0.3	59.2
deproteinized soybeen meal residue (after water washing)	81.7 ± 1.1	3.6 ± 1.2	17.4 ± 1.1	18.9 ± 1.2	1.1 ± 0.5	59.0

DLMR is composed mainly from insoluble carbohydrates (crude fiber and other non-starch polysaccharides), crude proteins, ash and residual oil. It's known that undehulled lupin seeds have a higher level of total insoluble polysaccharides versus soybean meal and it cannot be used in chicks diets and should be subjected to enzyme treatment [22].The composition of carbohydrate fraction of lupin raw material is very promising for obtaining fermented sugars better than from soy raw material. As known, insoluble carbohydrates of lupin seeds are composed mainly fromfiber, dextrines, hemicellulose, pectin substances, pentosans [11]. Insoluble carbohydrate fractions of yellow lupin seeds are represented mainly by arabinose, xylose, galactose, glucose and uronic acids [22]. Thus a significant part of fiber and hemicellulose compounds of DLMR could be subjected to enzymatic treatment with a high degree of bioconversion.

3.2. Biodegradation of DLMR *and ABE production*

The preliminary acid treatment of DLMR was performed to prepare the substrate for the subsequent enzymatic hydrolysis. Biodegradation of DLMR was based on a synergetic effect of cellulase and xylanase which affect cellulose, xylan and other hemicelluloses of lupin meal. Addition of α-amylase increased hydrolytic efficiency of the composition.The results are presented in Table 3.

Table 3. Parameters of substrate pretreatment process and ABE yield.

Type of enzyme	Dosage, %	Hydrolysis temperature °C	Carbohydrate concentration in hydrolysate, g L^{-1}	ABE yield, g L^{-1}
Cellolux-A	1	35	6.9 ± 0.1	3.1 ± 0.1
Cellolux-F	1	35	7.4 ± 0.2	3.2 ± 0.1
Multi-enzyme composition	1	35	9.6 ± 0.1	3.5 ± 0.1
Cellolux-A	2	35	7.2 ± 0.1	3.7 ± 0.1
Cellolux-F	2	35	8.2 ± 0.2	3.9 ± 0.1
Multi-enzyme composition	2	35	10.2 ± 0.1	4.2 ± 0.1

In case of usage of the multi-enzyme composition composed of cellulose, xylanase and amylase which was added to the substrate in a dose of 1 % by weight, the total carbohydrate concentration in hydrolysate was increased by around 28 % versus Cellolux-A,-F treatment and it reached concentration of 9.6 g L^{-1}.In case of increase of the multi-enzyme complex dosage up to 2 % of substrate weight, the bioconversion was improved up to 10.2 g L^{-1}. ABE yield increased in accordance with the increase of carbohydrate concentration in the range of 3.1 g L^{-1} to4.2 g L^{-1} ABE. Thus in the best embodiment after 72 hours of inoculation of *C. Beijerinckii* of hydrolyzed substrate with carbohydrate concentration 10.2 g L^{-1}ABE total content was 4.2 g L^{-1}.

U. S. Department of Agriculture researchers have proposed the ABE production technology from wheat straw. The ABE yield was observed [1, 2]. Hydrolysed DLMR hasthe concentration of carbohydrates comparable to the data of wheat straw and it could be regarded as a substrate for biofuel production toowith a higher economic attractiveness. The estimated economic impact can be assesed on the basis of raw material prices, technical maintenance costs, capital expenditures and so forth, see Table 4 [23].

Table 4. Total production cost.

Costs	€/kg ABE from wheat straw	€/kg ABE from DLMR
Raw materials	0.400	0.200
Labor	0.020	0.020
Technical Maintenance	0.120	0.150
Others	0.070	0.073
Taxes	0.024	0.024
Capital charge	0.246	0.252
Total gross	0.880	0.720

3. Conclusion

The experimental data show, that ABE synthesis from DLMRin integrated system using chemical and enzymatic substrate pretreatment has good possibilities. The offered method provides the maximum cost-effective use of resources in the processing of plant raw material. Thus, it is possible to use food industry wastes with a high content of structural polysaccharides. Use of the optimized enzyme complex allows to achieve a high degree of conversion of polysaccharides of plant origin into monosaccharides.

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